

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|   |  |  |   |
|---|--|--|---|
| (51) International Patent Classification <sup>6</sup> :<br>C07K 14/415, C12N 15/29, A61K 39/36,<br>C07K 14/735  |  | A1   | (11) International Publication Number: <b>WO 95/34578</b><br>(43) International Publication Date: 21 December 1995 (21.12.95) |
| (21) International Application Number: PCT/SE95/00724<br>(22) International Filing Date: 14 June 1995 (14.06.95)<br>(30) Priority Data:<br>9402089-8 14 June 1994 (14.06.94) SE<br>(71) Applicant (for all designated States except US): PHARMACIA<br>AB [SE/SE]; S-171 97 Stockholm (SE).<br>(72) Inventors; and<br>(75) Inventors/Applicants (for US only): BALL, Tanja [AT/AT];<br>Myrthengasse 5/4, A-1070 Wien (AT). VRTALA, Susanne<br>[AT/AT]; Schenkendorfgasse 14-16/1/9, A-1210 Wien<br>(AT). SPERR, Wolfgang [AT/AT]; Krlaseegasse 9, A-1190<br>Wien (AT). VALENT, Peter [AT/AT]; Schulgasse 7/18, A-<br>1170 Wien (AT). SUSANI, Markus [AT/AT]; Laufenstrasse<br>30, A-5020 Salzburg (AT). KRAFT, Dietrich [AT/AT];<br>Rebenweg 1/18/1, A-1170 Wien (AT). VALENTA, Rudolf<br>[AT/AT]; Beethovenstrasse 18, A-2604 Theresienfeld (AT).<br>LAFFER, Sylvia [AT/AT]; Krichbaumgasse 42/4, A-1120<br>Wien (AT).<br>(74) Agents: BERGANDER, Håkan et al.; Pharmacia AB, Patent<br>Dept., S-751 82 Uppsala (SE). |  | (81) Designated States: AU, CA, JP, US, European patent (AT, BE,<br>CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT,<br>SE).<br><br><b>Published</b><br><i>With international search report.<br/>Before the expiration of the time limit for amending the<br/>claims and to be republished in the event of the receipt of<br/>amendments.</i> |   |
| (54) Title: RECOMBINANT ALLERGEN, FRAGMENTS THEREOF, CORRESPONDING RECOMBINANT DNA MOLECULES,<br>VECTORS AND HOSTS CONTAINING THE DNA MOLECULES, DIAGNOSTIC AND THERAPEUTIC USES OF SAID<br>ALLERGENS AND FRAGMENTS   |  |  |   |
| (57) Abstract<br><br>A recombinant DNA molecule comprising a nucleotide sequence (I) which codes for a polypeptide displaying the antigenicity of one, two or more of the Phl p I epitope clones (28, 34, 41, 42, 43, 50, 52, 64, 80, 85, 86, 95, 97, 98, 103, 108, 109, 113, 114) with the amino acid sequences defined in figure 2 and preferably being derived from grasses or monocotyledonic plants, or a nucleotide sequence (II) which hybridizes with such a nucleotide sequence (I) under conditions of high stringency. Polypeptides displaying the antigenicity of one, two or more of the Phl p I epitope clones (28, 34, 41, 42, 43, 50, 52, 64, 80, 85, 86, 95, 97, 98, 103, 108, 109, 113, 114) with the amino acid sequences defined in figure 2. Recombinant expression vectors containing the recombinant molecule and host cells transformed with the vector. Diagnostic methods based on utilizing the polypeptides in immunoassays for humoral antibodies and cellular reactions.                |  |  |   |

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |                                       |    |                          |
|----|--------------------------|----|---------------------------------------|----|--------------------------|
| AT | Austria                  | GB | United Kingdom                        | MR | Mauritania               |
| AU | Australia                | GE | Georgia                               | MW | Malawi                   |
| BB | Barbados                 | GN | Guinea                                | NE | Niger                    |
| BE | Belgium                  | GR | Greece                                | NL | Netherlands              |
| BF | Burkina Faso             | HU | Hungary                               | NO | Norway                   |
| BG | Bulgaria                 | IE | Ireland                               | NZ | New Zealand              |
| BJ | Benin                    | IT | Italy                                 | PL | Poland                   |
| BR | Brazil                   | JP | Japan                                 | PT | Portugal                 |
| BY | Belarus                  | KE | Kenya                                 | RO | Romania                  |
| CA | Canada                   | KG | Kyrgyzstan                            | RU | Russian Federation       |
| CF | Central African Republic | KP | Democratic People's Republic of Korea | SD | Sudan                    |
| CG | Congo                    | KR | Republic of Korea                     | SE | Sweden                   |
| CH | Switzerland              | KZ | Kazakhstan                            | SI | Slovenia                 |
| CI | Côte d'Ivoire            | LI | Liechtenstein                         | SK | Slovakia                 |
| CM | Cameroon                 | LK | Sri Lanka                             | SN | Senegal                  |
| CN | China                    | LU | Luxembourg                            | TD | Chad                     |
| CS | Czechoslovakia           | LV | Latvia                                | TG | Togo                     |
| CZ | Czech Republic           | MC | Monaco                                | TJ | Tajikistan               |
| DE | Germany                  | MD | Republic of Moldova                   | TT | Trinidad and Tobago      |
| DK | Denmark                  | MG | Madagascar                            | UA | Ukraine                  |
| ES | Spain                    | ML | Mali                                  | US | United States of America |
| FI | Finland                  | MN | Mongolia                              | UZ | Uzbekistan               |
| FR | France                   |    |                                       | VN | Viet Nam                 |
| GA | Gabon                    |    |                                       |    |                          |

**RECOMBINANT ALLERGEN, FRAGMENTS THEREOF, CORRESPONDING RECOMBINANT  
DNA MOLECULES, VECTORS AND HOSTS CONTAINING THE DNA MOLECULES,  
DIAGNOSTIC AND THERAPEUTIC USES OF SAID ALLERGENS AND FRAGMENTS.**

---

5

The present invention relates to the title aspects of the major grass pollen allergen Phl p I and IgE-binding epitopes present in this allergen and corresponding haptens. The invention also relates to fragments, including IgE-binding haptens, from other  
10 grass and monocotyledonic plant allergens containing the IgE binding epitopes of Phl p I. The invention is primarily concerned with epitopes that normally are found in one or more group I allergens.

**15 Background to the invention**

Up to 20 % of the population in industrialized countries suffer from Type I allergic symptoms (rhinitis, conjunctivitis, asthma bronchiale) (Myamoto et al., 1992). The crosslinking of IgE which is bound to mast cells and basophils via the high affinity  
20 receptor FcεRI is the key event leading to release of biological mediators such as histamine (Segal et al., 1977). The crosslinking event by allergens represents, therefore, a potential target for therapy of Type I allergy. Such therapeutical approaches could either use portions of the IgE-  
25 molecule or other ligands, to interfere with the binding of IgE to the high affinity Fcε-receptor, or reagents to block the subsequent signal transduction cascade thus preventing the degranulation of mast cells and basophils (Dreskin et al., 1988). An additional possibility for specific therapy would be to use  
30 haptens derived from complete allergens which by binding to IgE monovalently could block the crosslinking of IgE (Valenta et al., 1993a). IgE-haptens could also be used to modulate the immune response or to induce tolerance by immunotherapy with a minimum of anaphylactic side effects. Haptens can be obtained from  
35 complete allergens by proteolytic digestion. However, this often results in a mixture of fragments and enzymes that are difficult

to characterize. Synthesis of peptides based on the amino acid sequence of the allergens, is an alternative approach. Recently a number of cDNAs coding for important allergens (Scheiner et al., 1992) were isolated which can be used to determine IgE-epitopes  
5 by molecular biological techniques.

Grass pollen allergy is spread world wide and according to the prevalence of grass pollen allergy it can be expected that 75 % of all allergic patients suffer from grass pollen allergy (Freidhoff et al., 1986).

10 Among the grass pollen allergic patients more than 90 % display IgE-reactivity with group I allergens (Freidhoff et al., 1986; Valenta et al., 1992).

The full amino acid sequences and nucleotide sequences of the major grass pollen allergens have been known for some time  
15 (timothy grass Phl p I (Laffer et al., 1993), rye grass (Lolium perenne) Lol p I (Perez et al., 1990; Griffith et al., 1991; University of Melbourne WO-A-9203550; Brunet C et al., International Symposium on Molecular Biology of Allergens and the Atopic Response, Quebec City, Canada, February 18-22, 1995;  
20 Lamontagne P et al., International Symposium on Molecular Biology of Allergens and the Atopic Response, Quebec City, Canada, February 18-22, 1995), and from rye from timothy grass (Phleum pratense) Sec c I (Laffer et al., unpublished data).

During the priority year the determination of clones 80, 97 and  
25 98 as carriers for a group I conserved IgE binding epitope has been described (Ball et al., 1994a, b, c; Laffer et al., 1994;).

#### Definition

The term IgE-hapten identifies short allergen fragments on  
30 which only one IgE-antibody with a given specificity is allowed to bind. A real IgE-binding hapten will give no histamine release because it contains the binding site for exclusively one IgE antibody. The term epitope in the context of the present invention refers to an IgE-epitope if not otherwise specified. An  
35 epitope may be located on either an IgE-hapten or a longer

polypeptide comprising several IgE-binding sites/epitopes. The term IgE preferentially refers to human IgE.

#### Objectives of the invention

- 5 The objectives of the invention are to provide simple, better and more reliable in vitro and in vivo tests for grass pollen allergy as well as improved therapeutic methods for this disease.

#### The invention

- 10 A first aspect of the invention is a recombinant DNA molecule comprising a nucleotide sequence (I) which codes for a polypeptide displaying the antigenicity of at least one of the Phl p I epitope clones 28, 34, 41, 42, 43, 50, 52, 64, 80, 85, 86, 95, 97, 98, 103, 108, 109, 113, 114 with the amino acid  
15 sequences defined in figure 2 and preferably being derived from grasses or monocotyledonic plants, or a nucleotide sequence (II) which hybridizes with such a nucleotide sequence (I) under conditions of high stringency. The recombinant DNA molecule comprises also degenerate variants of these nucleotide sequences.  
20 The recombinant DNA molecule may also contain a nucleotide sequence which codes for a polypeptide having antigenic crossreactivity and a high degree of homology, preferably > 50 % such as > 60 % or > 75 %, with Phl p I epitopes from grasses or other monocotyledonic plants, preferably those defined by the  
25 amino acid sequences given in figure 2.

A second aspect of the invention is a recombinant DNA expression vector or cloning system comprising an expression control sequence operatively linked to any of the recombinant molecules defined above.

- 30 A third aspect of the invention is a host cell containing a recombinant molecule or vector according to the first or second aspect, respectively.

- A fourth aspect of the invention is a recombinant or synthetic protein or polypeptide displaying the antigenicity of a Phl p I  
35 epitope, in particular comprising as an essential part a Phl p I epitope of at least one of the sequences set out in figure 2. The

protein or polypeptide may be fused to an additional polypeptide, such as  $\beta$ -galactosidase, GST or lambda cII protein or any other polypeptide that can be expressed as a fusion protein in prokaryotic or eukaryotic cells.

5 In the inventive poly/oligonucleotides and proteins/polypeptides, at least one of the sequences defined in figure 2 constitutes an essential part. For the poly-/oligonucleotides this means that each of them should not be longer than half of the DNA sequence coding for the full length Phl p I allergen and  
10 preferably containing a nucleotide sequence coding for at least one Phl p I epitope, such as being present in the Phl p I fragments specified in figure 2. The inventive oligo/poly-nucleotides chains are often shorter than 25 % of the DNA coding for the full length Phl p I allergen.

15 For the inventive proteins and polypeptides "essential part" means that each of them should not be longer than half of the full length Phl p I allergen and preferably also contain at least one Phl p I epitope, such as one or more of the epitopes defined by the fragments of full length Phl p I allergen specified in  
20 figure 2. The inventive proteins and polypeptides are often shorter than 25 % of the full length Phl p I allergen.

By the expression "a polypeptide displaying the antigenicity of at least one of the clones 28, 34, 41, 42, 43, 50, 52, 64, 80, 85, 86, 95, 97, 98, 103, 108, 109, 113, 114" is meant any peptide  
25 portion displaying at least one epitope defined by these clones and being recognizable immunologically. It can be envisaged that polypeptides exhibiting Phl p I epitopes may be derivatized to carry analytically detectable groups or water-soluble or water-insoluble solid phases suitable for immunoassays of antibodies  
30 directed against them, e.g. IgA, IgD, IgE, IgM or IgG antibodies. In aspects of the invention relating to in vitro diagnostics (see below) the inventive peptides may be a) linked to a water-insoluble phase by physical adsorption or a covalent bond, or b) conjugated covalently to an analytically detectable group  
35 (label).

The fifth aspect the invention is an in vitro method for diagnosing allergy to plant proteins by determining humoral antibodies directed towards the plant proteins. The allergies concerned are mostly against grass pollen. The relevant antibodies are mostly of the IgE class but IgG antibodies may also give information about the allergy. In general this method comprises contacting a body fluid sample derived from a patient with an inventive polypeptide. The amounts and conditions are selected so that an immune complex between the polypeptide and antibodies in the sample are formed in an amount that is a function of the amount of antibodies in the sample. The immune complex is then measured in a per se known manner.

More specifically a preferred method of the fifth aspect comprises contacting a body fluid sample containing the Igs concerned, e.g. IgG or IgE, with a polypeptide according to the invention and an anti-IgE antibody so as to form the immune complex containing peptide:IgE:anti-IgE. Normally either the peptide or the anti-IgE is linked to a solid phase that is insoluble or insolubilizable in the assay media so that the complex can be separated from the assay media. The determination step in these variants may be performed by use of an analytically detectable group (label) that either is covalently linked to the anti-IgE antibody (in case the peptide is linked to the solid phase) or to the peptide (in case the anti-IgE antibody is linked to the solid phase). In case IgG antibodies are to be determined anti-IgG replaces anti-IgE.

A sixth aspect of the invention is a method employing measuring, preferably in vitro, the cellular reaction against a Phl p I epitope. The method comprises using a recombinant or synthetic polypeptide as defined for the fourth aspect to stimulate the cellular reaction. Cellular reactions to be measured are histamine release and T cell proliferation (by  $^3\text{H}$  thymidine uptake).

The samples used in the above-mentioned methods are often derived from blood such as whole blood, serum and plasma,

although also other body fluids containing Igs may be used (tears etc).

Commonly accepted solid phase forms useful for immunoassays are walls of microtitre wells, spheres, rods, sheets, strips, pads etc. The solid phase may be porous or non-porous. The material in the solid phase may be a polymer selected among polysaccharides and their derivatives, for instance dextran, pullulan, agarose, cellulose etc, or synthetic polymers, preferably vinyl polymers, such as polyacrylamides, polyacrylates, polystyrene, polyvinyl alcohol etc. The polymers in question are often cross-linked, particularly in case the base polymer as such is water-soluble. Examples of analytically detectable groups are isotopes, enzymes, enzyme substrates, fluorophors, haptens, biotin etc.

A seventh aspect of the invention is a method for the treatment of a mammal, such as a human, which has a pollen allergy by administering a therapeutically effective amount of a recombinant or synthetic polypeptide as defined above. Illustrative examples of the therapeutic aspect of the invention are: a) passive therapy of effector organ (nose, conjunctiva, and lung) to prevent mediator release upon subsequent exposition to the complete allergen, and b) use of the peptides as safe tools for active immunotherapy because a single IgE epitope as such do not release mediators so that high doses can be applied. See further in the Discussion part below. The administration routes will be as commonly applied for current hyposensitization. The doses are likely to be in the  $\mu\text{g/ml}$ -range per kg body weight, i.e. 10-100  $\mu\text{g}$  per kg body weight.

#### EXPERIMENTAL PROCEDURES

A detailed description referring to the procedure for determining one IgE binding epitope encoded by the clones 80, 97 and 98. During the priority year the same method resulted in that further IgE binding epitopes were deduced (clones 28, 34, 41, 42, 43, 50, 52, 64, 85, 86, 95, 103, 108, 109, 113, 114). See Figure 2 for their specific sequences and positions within the Phl p I allergen.



**Construction of an epitope cDNA library from the randomly fragmented Phl p I cDNA.**

The cDNA fragment coding for Phl p I (Valenta et al., 1992; Laffer et al., 1993) was excised from plasmid pUC 18 and purified by preparative agarose gel electrophoresis (Sambrook et al., 1989; Ausubel et al., 1990). The cDNA was then randomly digested with DNase I (Sambrook et al., 1989; Ausubel et al., 1990) and fragments shorter than 400 bp was isolated by preparative agarose gel electrophoresis. The cDNA fragments were then end repaired with T4 polymerase (Boehringer Mannheim, Germany), linked with 5' phosphorylated 8-mer Eco R I linkers (Schmidheini, Windisch, Switzerland). After Eco R I digestion, linkers were removed using a nick column (Pharmacia Biotech AB, Uppsala, Sweden) and the inserts were ligated into dephosphorylated lambda gt11 arms (Pharmacia Biotech AB, Uppsala, Sweden). The phage DNA was then in vitro packaged using in vitro packaging extracts (Amersham, Buckinghamshire, U.K.). In the recombinant phage particles, the DNA inserts become fused to the gene for  $\beta$ -galactosidase.

20

**IgE-immunoscreening and analysis of epitope clones.**

100,000 phages of the Phl p I library were used to infect E. coli Y1090 at a density of 5,000 phages per plate (140 mm diameter). The synthesis of recombinant proteins was induced by overlaying the plates with nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) soaked in 10 mM IPTG (Huynh et al., 1985). 114 Phl p I epitope clones were isolated using serum IgE from a Phl p I allergic patient and  $^{125}$ I labeled rabbit anti-human IgE (Pharmacia Diagnostics, Uppsala, Sweden) as described (Breiteneder et al., 1989; Valenta et al., 1991; Vrtala et al., 1993a). The epitope clones were then characterized by hybridization with 3 synthetic oligonucleotides spanning the Phl p I cDNA (Oligo A: 5'GGG GGC TTG TCC ACA TCC TTG TAC CCG C3' bp 191-218, oligo B: 5'GGA GAG GTC GAA GTG GTA GGG G3' bp 372-393, Oligo C: 5'CCG CCA CCA CGT CTC CGT CGC CG3' bp 573-595). Nineteen clones that had hybridized with only one of the oligonucleotides

were tested for IgE-binding with sera from 12 Phl p I allergic patients. Clones 80, 97 and 98 bound IgE from most patients and were further tested for IgE-reactivity with sera from 90 grass pollen allergic patients as described (Valenta et al., 1992). For  
5 DNA sequence analysis, phage DNA was prepared, the epitope encoding cDNAs were excised with KpnI/SacI, subcloned into plasmid pUC 18 and both DNA strands were sequenced using lambda gt11 forward and reversed primers (Clontech, Palo Alto, USA) with <sup>35</sup>S dCTP (Sanger et al., 1977).

10

**Expression and purification of a immunodominant recombinant Phl p I epitope.**

The epitope encoded by clone 98 which bound IgE from 40 % of 90 grass pollen allergic patients and contained the shortest cDNA  
15 fragment was selected for purification. Recombinant clone 98 phage were used to infect lysogenic E. coli 1089 (Huynh et al., 1985). The Phl p I epitope encoded by clone 98 was expressed as a  $\beta$ -galactosidase fusion protein in liquid culture (Huynh et al., 1985) and was affinity purified using an anti- $\beta$ -galactosidase  
20 affinity column (Promega, Maddison, USA) as described (Vrtala et al., 1993a).  $\beta$ -galactosidase was obtained upon infection of E. coli Y1089 with empty lambda gt11 phage and purified in the same way.

**25 IgE-binding of natural timothy grass pollen allergens and recombinant Phl p I epitopes.**

Natural timothy grass pollen allergens were extracted from timothy grass pollen (Allergon, Valinge, Sweden) (Vrtala et al., 1993b) separated by SDS-PAGE (Laemmli et al., 1970) and  
30 transferred to nitrocellulose (Towbin et al., 1979). The recombinant Phl p I epitope (clone 98) and  $\beta$ -galactosidase were purified and also blotted on nitrocellulose. IgE from grass pollen allergic patients was used to detect the nitrocellulose blotted proteins as described (Jarolim et al., 1989) whereas IgE-  
35 binding to non-denatured recombinant epitopes was done using plaquelifts of phage clones as described elsewhere (Spitzauer et

al., 1993). IgE-binding synthetic peptides which were prepared by Cambridge Research Biochemicals, U.K. was measured by dot blot assays. One hundred nanograms to two mikrograms peptide per spot were dotted to nitrocellulose (Schleicher & Schuell, Dassel, Germany). Reactive peptides derived from other allergens and reactive sera were included as positive controls.

#### **In vitro histamine release from basophils of patients.**

Four grass pollen allergic patients with strong IgE-reactivity to group I grass pollen allergens were selected according to case history, serological testing, using RAST and immunoblotting with natural and recombinant grass pollen allergens and skin-prick test as described (Valenta et al., 1992). After informed consent was obtained heparinized blood samples were taken and granulocytes were prepared by dextran sedimentation (Valenta et al., 1989). Granulocytes were then incubated with increasing doses of natural timothy grass pollen allergens, anti-IgE mAb E-124-2-8 (positive control), the recombinant  $\beta$ -galactosidase fused Phl p I (clone 98) epitope and  $\beta$ -galactosidase (negative control), respectively. Liberated histamine expressed as percentage of total histamine was measured in the cell free supernatants by radioimmunoassay (Immunotech, Marseille, France) (Valenta et al., 1989).

To ensure that IgE-antibodies specific for clone 98 were present in the serum of the patient when histamine release was performed, the supernatants which were obtained from the granulocyte preparation were probed in parallel with nitrocellulose blotted timothy grass pollen allergens and the Phl p I (clone 98) epitope as described (Valenta et al., 1993b). The supernatants were from four grass pollen allergic individuals and from a non-allergic control individual. A buffer control without addition of a supernatant was also run.

#### **RESULTS**

**Isolation and characterization of an immunodominant IgE-epitope clone from Phl p I.**

100,000 phages from the Phl p I epitope library were screened using serum IgE from a grass pollen allergic individual with IgE-reactivity to group I grass pollen allergens. 114 IgE-binding phage clones were obtained and subsequently tested for hybridization with 3 synthetic oligonucleotides spanning the Phl p I cDNA. 19 clones which hybridized with only one oligonucleotide were further tested with serum IgE from 12 different Phl p I allergic patients. All tested patients displayed IgE-reactivity with clone 98 which therefore contained an immunodominant epitope. Clone 80 reacted with ten out of twelve Phl p I reactive patients and clone 97 with eleven out of twelve patients. When tested with serum IgE from 90 grass-pollen allergic patients which were selected according to case history, RAST (radioallergosorbent test) and skin prick tests, 40 % of the tested sera showed IgE-reactivity with clone 98 whereas 35 % reacted with clone 80 and clone 97 (data not shown).

The cDNAs of the three immunodominant epitope clones were sequenced and found to code for almost the same portion of the Phl p I molecule. Figure 2 shows the alignment of the deduced amino acid sequences of the epitope clones with the amino acid sequences of the major grass pollen allergen from timothy grass Phl p I (Laffer et al., 1993). The amino acid of clone 98 was aligned with the amino acid sequences of the other major grass pollen allergens from rye grass (*Lolium perenne*), Lol p I (Perez et al., 1990; Griffith et al., 1991), and from rye from timothy grass (*Phleum pratense*), Sec c I (Laffer et al., unpublished data). It was found that Clone 98 which reacted with IgE from all patients contained a 15 amino acid immunodominant IgE-epitope of Phl p I which is highly conserved among the other group I allergens and covers amino acid 101-115 of the mature Phl p I protein. The C-terminal portion of the peptide seemed to be critical for IgE-binding because clone 80 and 97 were less frequently recognized. Peptides of 12 amino acids length were synthesized according to the deduced amino acid sequence of Phl p

I with 3 amino acids overlaps, spanning the complete allergen. No reactivity of the patients' IgE with these peptides could be detected indicating that the critical length for IgE-binding of the clone 98 epitope is in the range between 13-15 amino acids (data not shown). Two sythetic peptides (D: aa 76-87, E: aa 117-129) which flanked the clone 98 IgE-epitope also did not bind IgE (data not shown).

**Comparison of different methods for the determination of B-cell epitopes.**

Different methods were used to determine IgE-epitopes (= B-cell epitopes) of Phl p I. Overlapping peptides (Geysen et al., 1987) with a length of 12 amino acids which were synthesized according to the deduced amino acid sequence of Phl p I were tested by dot blotting for reactivity with serum IgE from grass pollen allergic patients. In addition ten peptides covering regions of a predicted high antigenicity (Jameson et al., 1988) of the mature Phl p I allergen:

- peptide A: aa 1-17
- peptide B: aa 20-39
- peptide C: aa 44-69
- peptide D: aa 76-87
- peptide E: aa 117-129
- peptide F: aa 136-147
- peptide G: aa 151-159
- peptide H: aa 177-193
- peptide I: aa 199-209
- peptide J: aa 214-237)

were also tested for IgE-reactivity with 35 sera from grass pollen allergic patients by dot blotting. None of the peptides tested reacted with IgE from more than 5 % of the grass pollen allergic patients (data not shown).

Since the epitope mapping based on the use of synthetic peptides was unsuccessful, a recombinant mapping strategy was used (Mehra et al., 1986). An epitope expression cDNA library was constructed using randomly fragmented Phl p I cDNA. 114 IgE-

epitope clones could be isolated out of 100,000 phages which were screened with IgE from sera of patients. 19 epitope clones containing cDNAs that hybridized only with one oligonucleotide and, therefore, contained small Phl p I fragments were selected. 5 These clones were further tested with sera from 12 different grass pollen allergic patients to determine immunodominant IgE-epitopes. One prominent IgE-epitope encoded by clone 98 was isolated. This sequence was not predicted to be antigenic by computer analysis (Jameson et al., 1988) nor was it identified by 10 testing overlapping synthetic peptides spanning the complete Phl p I molecule (Geysen et al., 1987).

**IgE-binding capacity of recombinant Phl p I epitopes.**

Recombinant Phl p I epitopes were expressed as  $\beta$ -galactosidase 15 fusion proteins and tested for IgE-binding as native and denatured proteins.  $\beta$ -galactosidase produced by lambda gt11 phage without insert was used as a negative control. Native recombinant IgE-epitopes were obtained by overlaying plates of recombinant E. coli/phage with IPTG soaked membranes (Valenta et al., 1992) 20 whereas for the assays under denaturing conditions, IgE-epitopes were purified by affinity to anti- $\beta$ -galactosidase antibodies and were separated by denaturing SDS-PAGE (Laemmli et al., 1970) and electroblotted to nitrocellulose (Towbin et al., 1979). Clone 98 bound IgE from all 12 Phl p I allergic patients and when tested 25 with sera from 90 grass pollen allergic patients which were selected according to clinical criteria (case history, RAST and skin prick test) 40 % were found reactive.

Although clone 98 represented the immunodominant epitope some clones seemed to possess a higher IgE binding capacity. The 30 denatured immunoblotted clone 98 epitope also bound IgE from group I allergic patients. Compared with natural timothy grass pollen extracts a different intensity of IgE-binding was observed. This may be explained by the presence of group V allergens which comigrate with group I allergens in natural 35 extracts.  $\beta$ -galactosidase did not bind IgE in any of the two assays.

Identification of the immunodominant recombinant Phl p I IgE-epitope (clone 98) as a IgE-hapten by in vitro histamine release assays.

5 The recombinant Phl p I IgE-epitope encoded by clone 98 was tested for its capacity to release histamine from basophils of grass pollen allergic patients. Although all of the three patients displayed distinct IgE-reactivity to the nitrocellulose blotted recombinant Phl p I epitope, no histamine release was  
10 observed when the patients granulocytes were incubated with the purified epitope. A dose dependent and specific histamine release was measured when granulocytes from patients were incubated with natural grass pollen allergens and anti-IgE mAb (positive control) whereas no release was obtained upon incubation with  $\beta$ -  
15 galactosidase (negative control). One grass pollen allergic patient 98 epitope but with high levels of IgE against group V allergens was also included in the histamine release assays. This patient showed no histamine release with the clone 98 epitope whereas a dose dependent histamine release could be obtained with  
20 total grass pollen extracts that contained group V allergens.

#### DISCUSSION

The present study demonstrates the efficient determination of IgE-epitopes using recombinant techniques. The cDNA coding for  
25 the major grass pollen allergen, Phl p I (Valenta et al., 1992; Laffer et al., 1993), which is the target for IgE-antibodies of 90 % of the grass pollen allergic patients, and thus is the target for IgE-antibodies of up to 75 % of all allergic patients (Freidhoff et al., 1986; Valenta et al., 1992) was selected as  
30 starting material to construct an epitope expression cDNA library. Using IgE of grass pollen allergic patients, an immunodominant IgE-epitope clone containing a Phl p I peptide having a length of 15 amino acids was isolated.

The described 15 amino acids epitope was not predicted by a  
35 computer algorithm (Jameson et al., 1988) nor was it detected by overlapping peptide synthesis technology (Geysen et al., 1987).

The knowledge of IgE-epitopes is of particular importance because release of biological mediators such as histamine during the allergic effector reaction requires a divalent cross-link by allergens of IgE bound to mast cells and basophils (Segal et al., 1977). IgE-haptens derived from allergens contain only one IgE-epitope and thus cannot trigger allergic effector mechanisms unless they are polymerized. The immunodominant IgE-epitope which we derived from the major grass pollen allergen Phl p I was therefore purified and tested for its capacity to induce histamine release from the basophils of grass pollen allergic patients. Although in all experiments histamine release could be induced with natural timothy grass pollen allergens in a dose dependent way, no histamine release could be elicited with the recombinant Ph p I epitope which therefore represents an immunodominant IgE-hapten.

IgE-haptens may be useful for two therapeutical approaches of allergic disease: Haptens could be used to block mast cell- and basophil-bound IgE thereby directly inhibiting mediator release. Synthetic epitopes could be synthesized in large amounts and used directly in the effector organs (lung nasal mucosa, conjunctiva). Such an approach would however require the characterization of many different haptens according to the IgE-binding pattern of the patient. Using recombinant DNA techniques for the characterization of allergens and the determination of IgE-epitopes such an approach may be feasible. In addition, it is expected that due to extensive immunological crossreactivities among most allergens it might be possible to define a limited number of IgE-epitopes (Valenta et al., 1993a). As was demonstrated for Phl p I, IgE-epitopes can be determined by immunoscreening of expression libraries derived from cDNAs of allergens. A representative number of patients can then be tested for IgE-reactivity with recombinant epitope clones to obtain immunodominant structures. In a second step most of the additional relevant IgE-epitopes of each allergen have to be characterized. This might be possible because B-cell epitopes, unlike T-cell epitopes, assemble a larger conformation that also



has to be available on the surface of the allergen (Berzofsky et al., 1985; Chothia et al., 1991; Laver et al., 1990). thus it can be assumed that the diversity of B-cell epitopes may be much more restricted than that of T-cell epitopes.

5 In addition to the blocking of the allergic effector reaction, IgE-haptens could also be used to modulate IgE-responses by applying vaccination strategies which aim at the induction of immunological tolerance. Hyposensitization treatment of Type I allergies which is established world wide requires the increasing  
10 application of allergens by injection or oral administration. Although hyposensitizations is used successfully since 1911 (Noon et al., 1911), many patients undergoing this therapy suffer from severe side effects such as anaphylactic shock. The use of IgE-haptens derived from the allergens might contribute considerably  
15 to the improvement of this therapy by reduction of such side effects. Different other methods to modulate the IgE-response in allergic patients which are currently developed might also take advantage from the use of IgE-haptens.

In conclusion our study demonstrates that by use of recombinant  
20 techniques an immunodominant IgE-hapten from the major timothy grass pollen allergen Phl p I could be obtained. This allergen was selected as a model allergen because a high proportion (> 90 %) of all grass pollen allergic patients, and thus almost 75 % of all allergic patients show IgE-reactivity with this molecule.  
25 Although the obtained 10 amino acids epitope bound IgE from approximately 40 % of grass pollen allergic patients, it did not release histamine from the basophils of patients and may therefore be considered as a hapten. We are aware that in addition to the described Phl p I epitope certainly more epitopes  
30 from the same molecule and other allergens must be defined to block histamine release by saturating mast cells and basophils of allergic patients. However, the results encourage to continue with the characterization of IgE-haptens for their future use in specific therapy of allergic diseases.

35

**SUBSTITUTE SHEET**

## REFERENCES

1. Ausubel F M, In: Current protocols in Molecular Biology, Wiley, New York (1990)
2. Ball T et al., J. Biol. Chem. 269 (1994) 28323-28328
- 5 3. Ball et al., XV Annual Meeting of the European Academy of Allergology and Clinical Immunology, Stockholm, Sweden, June 26-July 1, 1994
4. Ball et al., 12th European Immunology Meeting, Barcelona, Spain, June 14-17, 1994
- 10 5. Brunet C et al., International Symposium on Molecular Biology of Allergens and the Atopic Response, Quebec City, Canada, February 18-22, 1995
6. Berzofsky J A, Science 229 (1985) 923-940
7. Breiteneder H et al., EMBO J. 8 (1989) 1935-1938
- 15 8. Chothia C, Current Opinion in Structural Biology 1 (1991) 53-59
9. Dreskin S C et al., J. Am. Med. Ass. 260 (1988) 1265-1268
10. Freidhoff L R et al., J. Allergy Clin. Immunol. 78 (1986) 1190-1201
- 20 11. Geysen H M et al., J. Immunol. Meth. 102 (1987) 259-274
12. Griffith I J et al., FEBS lett. 279 (1991) 210-215
13. Huynh T V et al., In: cDNA cloning, Oxford, IRL Press, 1 (1985) 49-78
14. Jameson B A et al., CABIOS 4 (1988) 181-186
- 25 15. Jarolim E et al., Allergy 44 (1989) 385-395
16. Laemmli U K, Nature (Lond.) 227 (1970) 680-685
17. Laffer S et al., J. Allergy Clin. Immunol (1993 in press)
18. Laffer et al., 12th European Immunology Meeting, Barcelona, Spain, June 14-17, 1994
- 30 19. Lamontagne P et al., International Symposium on Molecular Biology of Allergens and the Atopic Response, Quebec City, Canada, February 18-22, 1995
20. Laver W G et al., Cell 61 (1990) 553-556
21. Mehra V et al., Proc. Natl. Acad. Sci. USA 83 (1986) 7013-35 7017

SUBSTITUTE SHEET

22. Miyamoto T, In: Advances in Allergology and Clinical Immunology, Eds Godard P et al., The Parthenon Publishing Group-Carnforth, U.K. and New Jersey, USA, (1992) 343-347
23. Noon L, Lancet 1 (1911) 1572
- 5 24. Perez M et al., J. Biol. Chem. 265 (1990) 16210-16215
25. Sambrook J et al., In: Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press (1989)
26. Sanger F et al., Proc. Natl. Acad. Sci. USA 74 (1977) 5463-5468
- 10 27. Ferreira et al., In: Advances in Allergology and Clinical Immunology, Eds. Godard P et al., The Parthenon Publishing Group-Carnforth, U.K. and New Jersey, USA, (1992) 115-126
28. Segal D M et al., Proc. Natl. Acad. Sci. USA 41 (1977) 457-467
- 15 29. Spitzauer S et al., J. Allergy Clin. Immunol. (1993 in press???)
30. Towbin H et al., Proc. Natl. Acad. Sci. USA 76 (1979) 4350-4354
31. Valent P et al., Proc. Natl. Acad. Sci. USA 86 (1989) 5542-5546
- 20 32. Valenta R et al., Science 253 (1991) 557-560
33. Valenta R et al., Int. Arch. Allergy Appl. Immunol. 97 (1992) 287-294
34. Valenta R et al., In: Vaccines 93, Eds Ginsberg H S et al., Cold Spring Harbor Laboratory Press, New York, USA (1993a) 37-41
- 25 35. Valenta R et al., J. Allergy Clin. Immunol. 91 (1993b)
36. Vrtala S et al., J. Immunol. 151 (1993a) 4773-4781
37. Vrtala S et al., Int. Arch. Allergy Immunol 102 (1993b) 160-169
- 30 38. University of Melbourne (WO-A-9203550)

**SUBSTITUTE SHEET**

**SEQUENCES 1.** cDNA and deduced amino acid sequence of Phl p I epitope clones discovered up to the priority date (c80, c97, c98).

c80: CAC ATC ACC GAC GAC AAC GAG GAG CCC ATC GCC CCC TAC CAC TTC  
5           H   I   T   D   D   N   E   E   P   I   A   P   Y   H   F

GAC CTC TCC GGC CAC GCG  
D   L   S   G   H   A

10   C97: AAC GAG GAG CCC ATC GCC CCC TAC CAC TTC GAC CTC TCC GGC CAC  
         N   E   E   P   I   A   P   Y   H   F   D   L   S   G   H

GCG TTC GGG  
A   F   G

15

C98: GCC CCC TAC CAC TTC GAC CTC TCC GGC CAC GCG  
         A   P   Y   H   F   D   L   S   G   H   A

**SEQUENCES 2.** Alignment of the deduced amino acid sequences of all  
20   Phl p I IgE-epitope clones with the full amino acid sequence of  
the Phl p I allergen.

The clone number is at the right end. The full amino acid  
sequence of the Phl p I allergen is given by the longest lines.  
The underlined part corresponds to the signal peptide and is not  
25   present in the mature polypeptide.

H A S S S S V L L V V V L E A V E L G S A Y G I P K V P P G P N I T A T Y G D K W L D A K S T W Y G K P T G  
 I P K V P P G P N I T A T Y G D K W L D A K S T W Y G K P T G  
  
 A G P K D N G G A C G Y K D V D K P P F S G M T G C C G N T P I F K S G R G C G S C F E I K C T K P E A C S G  
 A G P K D N G G A C G Y K D V D K P P F S G M T G C C G N T P I F K S G R G C G N T  
 G Y K D V D K P P F S G M T G C C G N T P I F K S G R G C G S C F E I K C T K P E A C S 113  
 K P P F S G M T G C C G N T 45  
  
 E P V V V H I T D D N E E P I A P Y H F D L S G H A F G A M A K K G D E Q K L R S A G E L E L Q F R R V K C  
 P V V V H I T D D N E 43 I A P Y H F 34  
 H I T D D N E E P I A P Y H F D L S G H A 80  
 H I T D D N E E P I A P Y H F D L S G 114  
 T D D N E E P I A P Y H F D L S G H A F G A M A 95  
 T D D N E E P I A P Y H F 50  
 D N E E P I A P Y H F D L S G H A F G 97  
 N E E P I A P Y H F D L S G H 103  
 E P I A P Y H F D L S G H A F G M 98  
  
 K Y P E G T K V T F H V E K G S N P N Y L A L L V K Y V N G D G D V V A V D I K E K G K D K W I E L K E S W  
 H V E K G S N P N V L A L L V K Y V N G D G D V V A V 64  
  
 G A I W R I D T P D K L T G P F T V R Y T T E G G T K T E A E D V I P E G W K A D T S Y E S K 109  
 L T G P F T V R Y T T E G G T K T E A E D V I P E G W K A D T S Y E S K 86  
 G P F T V R Y T T E G G T K T E A E D V I P E G W K A D T S Y E S K 108  
 V R Y T T E G G T K T E A E D V I P E G W K A D T S Y E S K 42  
 R Y T T E G G T K T E A E D V I P E G W K A D T S Y E S K 52  
 T T E G G T K T E A E D V I P E G W K A D T S Y E S K 42

SUBSTITUTE SHEET

## P A T E N T   C L A I M S

1. A recombinant DNA molecule comprising a nucleotide sequence (I) which codes for a polypeptide displaying the antigenicity of one, two or more of the Phl p I epitope clones 28, 34, 41, 42, 43, 50, 52, 64, 80, 85, 86, 95, 97, 98, 103, 108, 109, 113, 114 with the amino acid sequences defined in figure 2 and preferably being derived from grasses or monocotyledonic plants, or a nucleotide sequence (II) which hybridizes with such a nucleotide sequence (I) under conditions of high stringency.
2. A recombinant DNA molecule according to any one of claims 1-2, comprising a nucleotide sequence which codes for a polypeptide having antigenic crossreactivity and a high degree of homology, preferably > 50 % such as > 60 % or > 75 %, with Phl p I epitopes from grasses or other monocotyledonic plants, preferably those defined by the amino acid sequences given in figure 2 for clones 28, 34, 41, 42, 43, 50, 52, 64, 80, 85, 86, 95, 97, 98, 103, 108, 109, 113, 114.
3. A recombinant DNA expression vector or cloning system comprising an expression control sequence operatively linked to a recombinant molecule defined in any one of claims 1-2.
4. A recombinant expression vector in which an expression control sequence is operationally linked to a coding nucleotide sequence which hybridizes with a Phl p I cDNA clone defined by the amino acids sequences given in figure 2, said hybridization taking place under conditions of high stringency.
5. A host cell containing a recombinant molecule or vector according to any one of claims 1-4.
6. A polypeptide displaying the antigenicity of at least one of the Phl p I epitopes defined by the amino acid sequences given in clones 28, 34, 41, 42, 43, 50, 52, 64, 80, 85, 86, 95, 97, 98,

**SUBSTITUTE SHEET**

103, 108, 109, 113, 114 of figure 2 or a Phl p I unique portion of these sequences.

7. A polypeptide according to claim 6 in which the synthetic  
5 protein or polypeptide is fused to an additional polypeptide.

8. A polypeptide according to claim 7 wherein said additional  
polypeptide is  $\beta$ -galactosidase, GST or lambda cII protein or any  
other polypeptide that can be expressed as a fusion protein in  
10 prokaryotic or eukaryotic cells.

9. Method of diagnosing allergy, preferably in vitro, to plant  
proteins in a patient which comprises contacting a body fluid  
sample, suspected of containing antibody against the plant  
15 protein, with a polypeptide defined in claims 6-8 under  
conditions permitting the formation of a complex containing the  
antibody and the polypeptide, whereafter the complex is measured  
and related to the amount of the antibody in the sample, an  
elevated level being taken as an indication of allergy against a  
20 plant protein comprising the polypeptide.

10. Method for measuring, preferably in vitro, the cellular  
reaction against a Phl p I epitope, in which a recombinant or  
synthetic protein or polypeptide according to any of claims 6-8  
25 is used to stimulate the cellular reaction.

11. Method of treatment of an animal which has a pollen allergy  
by administering a therapeutically effective amount of a  
recombinant or synthetic protein or polypeptide according to any  
30 of claims 6-8.

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 95/00724

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 14/415, C12N 15/29, A61K 39/36, C07K 14/735  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

REG, CA, BIOSIS, WPI, CLAIMS, GENESEQ SWISSPROT, EMBL, PIRONLY

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|-----------|---|-----------------------|
| P,X       | WO 9421675 A2 (IMMULOGIC PHARMACEUTICAL CORPORATION), 29 Sept 1994 (29.09.94)   | 1-8                   |
|           | --  |                       |
| X         | WO 9203550 A1 (THE UNIVERSITY OF MELBOURNE), 5 March 1992 (05.03.92)  | 1-8                   |
|           | --  |                       |
| X         | WO 9410314 A1 (THE UNIVERSITY OF MELBOURNE), 11 May 1994 (11.05.94)   | 1-8                   |
|           | --  |                       |
| X         | THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 265, No 27, Sept 1990, Mary Perez et al, "cDNA Cloning and Immunological Characterization of the Rye Grass Allergen Lol p I", page 16210 - page 16215 | 1-8                   |
|           | --  |                       |

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

6 October 1995

11-10-1995

Name and mailing address of the ISA/  
Swedish Patent Office  
Box 5055, S-102 42 STOCKHOLM  
Facsimile No. +46 8 666 02 86

Authorized officer

Carolina Gómez Lagerlöf  
Telephone No. +46 8 782 25 00



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE95/00724

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 9-11  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

28/08/95

International application No.

PCT/SE 95/00724

| Patent document<br>cited in search report |         | Publication<br>date | Patent family<br>member(s) |         | Publication<br>date |
|---|---------|---------------------|----------------------------|---------|---------------------|
| WO-A2-                                    | 9421675 | 29/09/94            | NONE                       |         |                     |
| WO-A1-                                    | 9203550 | 05/03/92            | AU-B-                      | 659801  | 01/06/95            |
|   |         |                     | AU-A-                      | 8408391 | 17/03/92            |
|   |         |                     | CA-A-                      | 2089735 | 18/02/92            |
|   |         |                     | EP-A, A-                   | 0576426 | 05/01/94            |
|   |         |                     | JP-T-                      | 5509230 | 22/12/93            |
| WO-A1-                                    | 9410314 | 11/05/94            | NONE                       |         |                     |